

LAMININ CHAINS: DIAGNOSTIC USES

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CROSS REFERENCE

This application claims the benefit of the filing date of U.S. Provisional Application No. 60/175,005, filed January 7, 2000. This application is also a continuation-in-part of U.S. Application Serial No. 09/663,147, filed on September 15, 2000, which is a continuation of U.S. Application Serial No. 08/800,593 filed February 18, 1997, now U.S. Patent No 6,143,505, which is a divisional of U.S. Application Serial No. 08/317,450 filed October 4, 1994, now U.S. Patent No. 5,660,982.

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BACKGROUND OF THE INVENTION

Laminins are a family of basement membrane proteins which function in cell differentiation, adhesion, and migration, in addition to being true structural components (Tryggvason K, Curr. Opin. Cell Biol., 1993, 5:877-882, this and all following references are hereby incorporated by reference). The laminin molecule is a cross-shaped heterotrimer consisting of one heavy chain (≈ 400 kd) and two light chains, β and γ (130-200 kd) (nomenclature according to Burgeson et al., Matrix Biol., 1994, 14:209-211). Laminins exist as several isoforms each having a unique combination of α , β and γ chains. Thus far, ten genetically distinct laminin chains, $\alpha 1 - \alpha 5$, $\beta 1 - \beta 3$ and $\gamma 1 - \gamma 2$ are known.

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In the laminin molecule the three chains are associated through a carboxyl terminal coiled coil (long arm), most of the chains having a free amino terminal short arm. Additionally, all the α chains have a large globular G domain at the carboxyl terminus. Laminin can contribute to the structural framework of the basement membrane, but it is also believed to have a role in cell differentiation, proliferation, adhesion and migration (Timpl, R. & Brown, J. C. (1994) Matrix Biol. 14: 275-81, Yurchenco, P.D. & O'Rear, J.J. (1994) Curr. Opin. Cell. Biol. 6: 674-81). Many of the laminin chains have tissue- and cell-specific distribution which may vary between different developmental stages, indicating specific functions for the various chains and isoforms. Evidence for tissue-specific roles of some of the laminin chains has come from

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identification of mutations in the $\alpha 2$ chain gene in muscular dystrophies in mouse and man (Xu, H., Wu, X.R., Wewer, U.M. & Engvall, E. (1994) *Nature Genet.* 8: 297-301; Heibling-Leclerc, A., Zhang, X., Topaloglu, H., Cruaud, C., Tesson, F., Weissenbach, J., Tome', F., Schwartz, K., Fardeau, M., Tryggvason, K. & Guicheney, P. (1995) *Nature Genet.* 11: 216-218; Nissinen, M., Heibling-Leclerc, A., Zhang, X., Evangelista, T., Topaloglu, H., Cruaud, C., Weissenbach, J., Fardeau, M., Tome', F. M.S., Schwartz, K., Tryggvason, k. & Guicheney, P. (1996) *Am. J. Hum. Genet.* 58: 1177-1184), as well as in the genes for the $\alpha 3$, $\beta 3$ and $\gamma 2$ chains in epidermolysis bullosa (Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K. & Uitto, J. (1994a) *Nature Genet.* 6: 293-297; Pulkkinen, L., Christiano, A.M., Gerecke, D., Wagman, D.W., Burgeson, R.E., Pittelkow, M.R. & Uitto, J. (1994b) *Genomics* 24: 357-60; Aberdam, D., Galliano, M.F., Vailly, J., Pulkkinen, L., Bonifas, J., Christiano, A. M., Tryggvason, K., Uitto, J., Epstein, E. J., Ortonne, J. P. & Meneguzzi, G. (1994) *Nature Genet.* 6: 299-304; Kivirikko, S., McGrath, J. A., Baudoin, C., Aberdam, D., Ciatti, S., Dunnill, M. G. S., McMillan, J. R., Eady, R. A. J., Ortonne, J-P., Meneguzzi, G., Uitto, J. & Christiano, A. M. (1995) *Hum. Mol. Genet.* 4: 959-962; Vidal, F., Baudoin, C., Miquel, C., Galliano, M-F., Christiano, A. M., Uitto, J., Ortonne, J-P. & Meneguzzi, G. (1995) *Genomics* 30: 273-280).

Laminin-5, is a unique subepithelial basement membrane isoform with the molecular formula $\alpha 3:\beta 3:\gamma 2$ chains (Burgeson, R. E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H., Martin, G.R., Meneguzzi, G., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., & Yurchenco, P.D. (1994) *Matrix Biol.* 14: 209-211). Determination of the primary structure of the human $\alpha 3$, $\beta 3$ and $\gamma 2$ chains has revealed that all these chains are truncated in the short arm relative to the corresponding chains of laminin-1 (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) *J. Cell Biol.* 119: 679-693; Ryan, M.C., Tizard, R., VanDevanter, D.R. & Carter, W.G. (1994) *J. Biol. Chem.* 269: 22779-22787; Gerecke, D.R., Wagman, D.W., Champlaud, M.F. & Burgeson, R.E. (1994) *J. Biol. Chem.*). Additionally, the $\gamma 2$ chain exists in two forms differing in the length of their carboxyl terminal end due to alternative splicing Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) *J. Cell Biol.* 119: 679-693; Ryan, M.C., Tizard,

R., VanDevanter, D.R. & Carter, W.G. (1994) *J. Biol. Chem.* 269: 22779-22787; Gerecke, D.R., Wagman, D.W., Champliand, M.F. & Burgeson, R.E. (1994) *J. Biol. Chem.*; Airenne, T., Haakana, H., Sainio, K., Kallunki, T., Kallunki, P., Sariola, H. & Tryggvason, K. (1996) *Genomics* 32: 54-64). Immunolocalization of the laminin-5 protein (previously termed kalinin, nicein or epiligrin) to anchoring filaments (Verrando, P., Hsi, B., Yeh, C., Pisani, A., Serieys, N., & Ortonne, J. (1987) *Exp. Cell Res.* 170:116-128; Carter, W.G., Ryan, M.C. & Gahr, P.J. (1991) *Cell* 65: 599-610; Rousselle, P., Lunstrum, G.P., Keene, D.R. & Burgeson, R.E. (1991) *J. Cell Biol.* 114: 567-576) as well as epithelium-specific expression of the $\gamma 2$ chain (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) *J. Cell Biol.* 119: 679-693) already implied its role as an epithelial attachment component. The adhesion properties of laminin-5 have been demonstrated in several cell attachment studies (Carter, W.G., Ryan, M.C. & Gahr, P.J. (1991) *Cell* 65: 599-610; Rousselle, P., Lunstrum, G.P., Keene, D.R. & Burgeson, R.E. (1991) *J. Cell Biol.* 114: 567-576; Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, L.M.H., Falcioni, R., Kennel, S.J., Aplin, J.D., Baker, J., Loizidou, M. & Garrod, D. (1991) *J. Cell Biol.* 113: 907-917; Niessen, C.M., Hogervorst, F., Jaspars, L.H., De Melker, A.A., Delwel, G.O., Hulsman, E.H., Kuikman, I. & Sonnenberg, A. (1994) *Exp. Cell. Res.* 211: 360-367; Rousselle, P. & Aumailley, M. (1994) *J. Cell Biol.* 125:205-214). The adhesive function of laminin-5 has been shown to be mediated through $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins (Carter, W.G., Ryan, M.C. & Gahr, P.J. (1991) *Cell* 65: 599-610; Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, L.M.H., Falcioni, R., Kennel, S.J., Aplin, J.D., Baker, J., Loizidou, M. & Garrod, D. (1991) *J. Cell Biol.* 113: 907-917; Rousselle, P. & Aumailley, M. (1994) *J. Cell Biol.* 125:205-214). Direct evidence for the crucial role of laminin-5 for epithelial cell attachment has come from the identification of mutations in the genes of all the subunit chains (Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K. & Uitto, J. (1994) *Nature Genet.* 6: 293-297; Pulkkinen, L., Christiano, A.M., Gerecke, D., Wagman, D.W., Burgeson, R.E., Pittelkow, M.R. & Uitto, J. (1994b) *Genomics* 24: 357-60; Aberdam, D., Galliano, M.F., Vailly, J., Pulkkinen, L., Bonifas, J., Christiano, A. M., Tryggvason, K., Uitto, J., Epstein, E. J., Ortonne, J. P. & Meneguzzi, G. (1994) *Nature Genet.* 6: 299-304; Kivirikko, S., McGrath, J. A., Baudoin, C., Aberdam, D., Ciatti, S., Dunnill, M. G. S., McMillan, J. R.,

Eady, R. A. J., Ortonne, J-P., Meneguzzi, G., Uitto, J. & Christiano, A. M. (1995) *Hum. Mol. Genet.* 4: 959-962; Vidal, F., Baudoin, C., Miquel, C., Galliano, M-F., Christiano, A. M., Uitto, J., Ortonne, J-P. & Meneguzzi, G. (1995) *Genomics* 30: 273-280) in the Herlitz's variant of junctional epidermolysis bullosa, a lethal skin blistering disease
 5 caused by disruption of the epidermal-dermal junction. One and possibly the only cell adhesion site of laminin-5 has been localized to the long arm (Rousselle, P. & Aumailley, M. (1994) *J. Cell Biol.* 125:205-214; Rousselle, P., Golbik, R., van der Rest, M. & Aumailley, M. (1995) *J. Biol. Chem.* 270:13766-13770). However, a mutation in one junctional epidermolysis bullosa patient causing an in-frame deletion of 73 residues
 10 from domains III and IV of the short arm of the laminin γ 2 chain indicates a role for this part of the chain for the anchorage of epithelial cells to the extracellular matrix (Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K. & Uitto, J. (1994) *Nature Genet.* 6: 293-297).

By in situ hybridization the γ -2 chain was found to be expressed in epithelial
 15 cells of many embryonic tissues such as those of skin, lung, and kidney (Kallunki et al., 1992, supra.), and antibodies to kalinin/laminin 5, react with basement membranes of the same tissues (Rousselle et al., 1991, supra.; Verrando et al., *Lab. Invest.*, 1991, 64:85-92).

The different laminin chains have been shown to have quite varying tissue
 20 distribution as determined by immunohistological studies, Northern, and in situ hybridization analyses. For example, the A and M chains on the one hand, and the B1 (β -1) and S (β -2) chains on the other, have been shown to be mutually exclusive (see for example Vuolteenaho et al., *J. Cell Biol.*, 1994, 124:381-394). In vitro studies have indicated that laminin mediates a variety of biological functions such as stimulation of
 25 cell proliferation, cell adhesion, differentiation, and neurite outgrowth. The cellular activities are thought to be mediated by cell membrane receptors, many of which are members of the integrin family (Ruoslahti, E. *J. Clin. Invest.*, 1991, 87:1-5; Mecham, R. P. *FASEB J.*, 1991, 5:2538-2546; Hynes, R. *Cell*, 1992, 69:11-25). Recently a new nomenclature for describing laminins has been agreed to as in the following Table 1
 30 (after Burgeson et al., 1994, supra.):

TABLE 1**Laminin Chains and Genes**

New	Previous	Gene
$\alpha 1$	A, Ae	LAMA1
$\alpha 2$	M, Am	LAMA2
$\alpha 3$	200 kDa	LAMA3
$\beta 1$	B1, B1e	LAMB1
$\beta 2$	S, B1s	LAMB2
$\beta 3$	140 kDa	LAMB3
$\gamma 1$	B2, B2e	LAMC1
$\gamma 2$	B2t	LAMC2

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Heterotrimers of Laminin

New	Chains	Previous
Laminin-1	$\alpha 1\beta 1\gamma 1$	EHS laminin
Laminin-2	$\alpha 2\beta 1\gamma 1$	merosin
Laminin-3	$\alpha 1\beta 2\gamma 1$	s-laminin
Laminin-4	$\alpha 2\beta 2\gamma 1$	s-merosin
Laminin-5	$\alpha 3\beta 3\gamma 2$	kalinin/nicein
Laminin-6	$\alpha 3\beta 1\gamma 1$	k-laminin
Laminin-7	$\alpha 3\beta 2\gamma 1$	ks-laminin

Cell migration is one of the biological functions proposed for laminin (Timpl, R. & Brown, J. C. (1994) *Matrix Biol.* 14: 275-81). Cellular movement is required for various physiological and pathological processes, such as during embryogenesis, wound healing, angiogenesis and tumor invasion. Immunohistochemical and *in situ* hybridization studies have shown induction of laminin-5 expression in migrating keratinocytes during wound healing (Ryan, M.C., Tizard, R., VanDevanter, D.R. & Carter, W.G. (1994) *J. Biol. Chem.* 269: 22779-22787; Larjava, H., Salo, T., Haapasalmi, K., Kramer, R. H. & Heino J. (1993) *Clin. Invest.* 92: 1425-1435; Pyke, C., Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K. & Tryggvason, K (1994) *Am. J. Pathol.* 145: 782-791). The $\gamma 2$ chain of laminin-5 has also been shown to be strongly expressed in malignant cells located at the invasion front of several human carcinomas, as determined by *in situ* hybridization and immunohistochemical staining

(Pyke, C., Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K. & Tryggvason, K (1994) Am. J. Pathol. 145: 782-791; Pyke, C., Salo, S., Ralfkiaer, E., Romer, J., Dano, K. & Tryggvason, K. (1995) Cancer Res. 55: 4132-4139). Since laminin-1 has been found to inhibit keratinocyte migration *in vitro* (Woodley, D. T., Bachmann, P. M. & O'Keefe, E. J. (1988) J. Cell. Physiol. 136: 140-146), and as the laminin $\alpha 1$, $\beta 1$ and $\gamma 1$ chains are only weakly expressed throughout cancerous areas with no apparent correlation to sites of invasion, laminin-5 has been proposed to have a role in the migration event (Pyke, C., Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K. & Tryggvason, K (1994) Am. J. Pathol. 145: 782-791).

SUMMARY OF THE INVENTION

The instant invention provides for methods of detecting kalinin/laminin 5 expression in tissue comprising detecting a signal from assayed tissue, such signal resulting from specifically hybridizing tissue with an effective amount of a nucleic acid probe, which probe contains a sense or antisense portion of kalinin/laminin 5 gamma-2 nucleic acid sequence (Kallunki et al., 1992, supra.). In particular, where the nucleic acid probe is DNA, RNA, radio-labeled, enzyme labeled, chemiluminescent labeled, avidin or biotin labeled, derived from human kalinin/laminin 5 gamma-2 nucleic acid sequence, incorporated into an extrachromosomal self-replicating vector, a viral vector, is linear, circularized, or contains modified nucleotides. In the preferred embodiment the probes are linearized specific regions of the γ -2 gene.

The instant invention also provides for methods for detecting the presence of invasive cells in tissue comprising detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a nucleic acid probe, which probe contains a sense or antisense portion of kalinin/laminin 5 γ -2 nucleic acid sequence (Kallunki et al., 1992, supra.). In particular, where the nucleic acid probe is DNA, RNA, radio-labeled, enzyme labeled, chemiluminescent labeled, avidin or biotin labeled, derived from human kalinin/laminin 5 gamma-2 nucleic acid sequence, incorporated into an extrachromosomal self-replicating vector, a viral vector, is linear, circularized, or contains modified nucleotides. In the preferred embodiment the probes are linearized specific regions of the γ -2 gene. The instant method also provides for the diagnosis of the absence of γ -2 chain expression, useful for the monitoring of therapies,

and the progress of malignant cell transformation leading to accurate determination of the extent of invasive cell activity.

The instant invention further provides for a method for detecting kalinin/laminin 5 expression in tissue comprising detecting a signal from assayed tissue, such signal
5 resulting from contacting tissue with an effective amount of a labeled probe, which probe contains an antibody immunoreactive with a portion of kalinin/laminin 5 gamma-2 protein.

Further provided is a method for detecting invasive cells in tissue comprising
10 detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a labeled probe, which probe contains an antibody immunoreactive with a portion of kalinin/laminin 5 gamma-2 protein. Also provided is a method for detecting kalinin/laminin 5 in tissue comprising detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a
15 labeled probe, which probe contains an antibody immunoreactive with a portion of kalinin/laminin 5 gamma-2 protein. Thus the method of the instant invention provides for the absence of such signal as diagnostic for the absence of invasive cells.

Further, the present invention provides for a method of using the laminin-5 molecule to promote adhesion of cultured epithelial and carcinoma cells.

Additionally, the present invention is directed to a method for blocking migration
20 of cells using antibodies against the γ -2 chain of the laminin-5 molecule. Evidence for the relationship of γ 2 chain expression with cell migration was obtained by the identification of an enhancer element in the *LAMC2* gene in studies on promoter-reporter gene constructs in transgenic mice.

25 BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-D shows in situ hybridization of a specimen of colon adenocarcinoma for γ -2 chain mRNA using a S-35 labeled anti-sense RNA probe derived from plasmid pbb2r-02. Magnification: 1A x 100; 1B-1D x 640.

~~**FIGS. 2A-D** shows in situ hybridization for γ -2 chain mRNA on sections of ductal mammary carcinoma (2A), malignant melanoma (2B), squamous cell carcinoma of the skin (2C-2D), and squamous cell carcinoma of the vulva (2E-2G). Magnification: 2C x 100, all others x 640. Photos marked by plain letter ie. X, show in situ hybridization~~

results for γ -2 chain mRNA on stained sections. Photos marked by the apostrophe letter, ie. X', are the dark field images of the respective photomicrographs.

FIGS. 3A,A' is incisionally wounded mouse skin (72 hours after wounding) showing signal for γ -2 chain in keratinocytes at the leading edge of the migrating epithelium (curved arrow). Magnification: x 640. FIG. 3A is a photo of in situ hybridization on a stained section showing γ -2 chain signal. FIG. 3A' is a photo showing the dark field image of 3A.

FIGS. 4A-B shows the nucleic acid sequence for the γ -2 chain cDNA and the derived amino acid sequence. FIG. 4A is the full cDNA for the 5,200 base pair sequence, available from EMB/GenBank/DDBJ under the accession number Z15008. FIG. 4B is the nucleotide and derived amino acid sequence of the alternative 3' end sequence from cDNA clones providing a sequence of 4,316 base pairs, available from EMB/GenBank/DDBJ under the accession number Z15009. (Kallunki et al.,1992, *supra*.) ~~SEQ ID NOS: 12,13,14 & 15.~~

FIG 5. Characterization of laminin-5 and recombinant laminin γ 2 chain prepared and used in this study. HaCat cell culture medium proteins and the recombinant laminin γ 2 chain extracted from baculovirus-infected insect cells were purified as described in *Materials and Methods* and studied on 6 % polyacrylamide gels. (A) Silver stained laminin-5 (LAM5) from HaCat cells was resolved into two major bands of 165 kDa and 140-155 kDa. Additionally, a weak band of 105 kDa could be observed. These bands correspond in size to those of the α 3 (165 kDa), γ 2+ β 3 (155 kDa+140 kDa) and processed γ 2 (105 kDa) chains of laminin-5. The recombinant γ 2 chain (rec γ 2) produced by the baculovirus system showed a major silver stain band of about 155 kDa).

FIG. 6. Efficiency of human laminin-5 and recombinant human laminin γ 2 chain for attachment of HaCat keratinocytes and KLN-205 squamous carcinoma cells *in vitro*. The attachment efficiency was compared with the efficiency with which the cells bound to laminin-1. Substrate concentrations (10 μ g/ml) providing maximum attachment to laminin-1 and laminin-5 were used. The results are presented as means \pm SD calculated from at least four duplicate series, the values for laminin-1 were given the arbitrary value of 100 %.

FIGS. 7A-B. Effects of polyclonal γ 2 chain antibodies on the migration of KLN-205 squamous carcinoma cells in Boyden and Transwell chamber assays of migration. (7A)

The two compartments of the chemotactic Boyden chambers were separated by a type IV collagen coated porous Nucleopore filter (pore size 8 μ m). The cells (1×10^5) in MEM containing 0.1 % BSA were placed in the upper compartment, and laminin-1 (+/-) or fibronectin (-/+) in MEM containing 0.1 % BSA were added as chemoattractants to the lower compartment. IgG against $\gamma 2$ chain domains III, I/II or preimmune IgG was added to the upper compartment with the cells at a concentration of 20 μ g/ml. After 8-hour incubation at 37° C the filters were removed and migration of cells to the lower surface of the filter were quantitated. The data are expressed as percentage of migrated cells (+/- SD (bars)) per high power field, setting migration in the presence of pre-immune IgG as 100 %. Cells were counted in ten randomly selected high power fields to triplicate assays. (7B), Effects of exogenous laminin-5 on cell migration in a Transwell assay. The lower side of the membrane was coated with EHS type IV collagen, and the lower compartment was filled with 2.5 μ g/ml laminin-5 as a chemoattractant. Pre-immune IgG, IgG against the $\gamma 2$ chain domains III or I/II were added to the upper chamber containing the cells. Following 16 hour incubation the cells were fixed and cells at the lower side of the membrane were counted (12 fields +/- SD).

FIG. 8. *LAMC2* Promoter-reporter gene constructs used for studies of expression in transgenic mice. *Top*, nucleotide sequence of the 5' region of the *LAMC2* gene and its immediate upstream region (SEQ. ID. NO.:20). The *bent arrow* indicates the site of transcription initiation. The GATAA motif is *boxed* and the AP-1, Sp1 and CTF motifs are *underlined*. The translation initiator codon ATG in exon 1 for methionine is indicated by a *double underline*. The first amino acids of the protein are shown with the single letter code beneath the corresponding codons. The large *boxed* area represents the sequence cloned into construct HH-2. *Bottom*, schematic illustration of the two *LAMC2-LacZ* reporter gene constructs HH-1 and HH-2 used in transgenic mice.

FIGS. 9A-D. Expression of *LAMC2-LacZ* reporter gene constructs in 15.5-day-old transgenic mouse embryos. (9A), Staining of the whole 15.5-day-old embryo reveals highly restricted expression of β -galactosidase as observed in some hair follicles and some regions of skin and testicles (arrow). Construct HH-2. (9B), Scattered epithelial cells of hair follicles are positive. Construct HH-1. (9C), Scattered positive epithelial cells of *ductus deferens*. Construct HH-1. (9D), Some positive epithelial cells of skin. Construct HH-2.

FIGS. 10A-D. Expression of shorter *LAMC2-LacZ* reporter gene construct HH-2 in adult transgenic mouse tissues. (10A), Regionally positive epithelial cells of the epidermis (*ep*) and hair follicles (*arrow*). (10B), Scattered positive epithelial cells in *ductus deferens*. (10C and 10D), Expression can be seen in some areas of the gastric mucosa. Note that the expression can be localized to epithelial cells of both the surface and in the gastric pits.

FIGS. 11A-D. Immunolocalization of laminin-5 $\gamma 2$ chain and expression of *LAMC2-LacZ* reporter gene constructs in dorsal incision skin wounds in transgenic mice. (11A), Immunostaining localizes the $\gamma 2$ chain to the basement membrane (*arrow*) beneath keratinocytes of the uninjured epithelium (*ep*) at the edges of the 3-day-old wound. (11B), Immunostaining reveals the presence of the $\gamma 2$ chain in the entire newly formed basement membrane deposited under the epithelium (*ep*) covering the 7-day-old incision wound. (11C), Expression of the *LAMC2-LacZ* reporter gene construct HH-2 in keratinocytes migrating from edges of the 3-day-old incision wound (*arrows*) during the initial phase of re-epithelialization. Only minor expression is observed in keratinocytes of the uninjured epithelium. (11D), Keratinocytes of the new epithelium covering the entire 7-day-old incision wound exhibit intense expression of the reporter gene.

DETAILED DESCRIPTION OF THE INVENTION

Epidermolysis bullosa (EB) is a group of mechano-bullous disorders characterized by fragility of the skin and mucous membranes (see Lin & Carter eds., Epidermolysis bullosa. Basic and clinical aspects, 1992, Springer Verlag, N.Y.; Fine et al., J. Am. Acad. Dermatol., 1991, 24:119-135). The junctional forms of EB (JEB) are characterized by tissue separation at the level of the lamina lucida within the dermal-epidermal basement membrane, and no specific mutation had yet to be reported. Recently it has been proposed that the genes for a lamina lucida protein kalinin/nicein/epiligrin may be a candidate in some forms of JEB (Verrando et al., 1991, supra.). Several lines of evidence suggest that anchoring filament proteins could be defective in some forms of JEB. First, attenuation or absence of immunoreactivity with anti-kalinin(epiligrin) antibodies has been noted in the skin of patients with the most severe (Herlitz) type of JEB. The immunofluorescence staining patterns may be of prognostic value in classifying JEB, and these immunoreagents have been used for

prenatal diagnosis of JEB using fetal skin biopsy specimens. Second, the kalinin/laminin 5 γ -2 chain is expressed in epithelial cells of the skin, trachea and kidneys, tissues which are frequently affected by JEB.

5 Since the majority of cases are of the generalized (Herlitz) phenotype (H-JEB), JEB patients have been classified into Herlitz and non-Herlitz types. Clinical features of H-JEB include mechanical fragility of the skin, with widespread blistering and erosions, rapid deterioration and neonatal death, often from sepsis. Longterm survival is rare.

10 Efforts to identify the basic defect in JEB began with the observation that a monoclonal antibody that binds to the lamina lucida of the epidermal basement membrane zone of normal skin, fails to react with the lamina lucida of H-JEB skin (Verrando et al., 1991, supra.). The antigen recognized by this antibody was purified from keratinocyte culture medium and termed BM600/nicein. Keratinocytes cultured from the skin of H-JEB patients attach poorly to substrate and fail to accumulate immunologically detectable nicein. Further experiments with antibodies specific for the 15 α -3 chain of nicein, demonstrated that they were capable of inducing the rounding and detachment of adherent keratinocytes without affecting fibroblasts (Rousselle et al., 1991, supra.). Thus the correlation in vivo and in vitro of the dermoepidermal separation with deficient nicein/kalinin/laminin 5 immunoreactivity and the separation induced by 20 anti-nicein antibody have made the genes encoding this protein strong candidates for the site of H-JEB mutations.

The importance of the γ -2 chain of nicein/kalinin/laminin 5 in JEB, and epithelial tissues prompted the investigation into the role such adhesion contacts between epithelial cells may play in abberant cells. Of primary interest was the role γ -2 25 chain of nicein/kalinin/laminin 5 abberant expression may play in cancer tissue, and a possible role in cancer dissemination.

It has been recently shown that in colon adenocarcinoma, a significant positive correlation between the degree of tumor budding and the recurrence of tumors following curative surgery exists, and that this fact is likely to reflect a higher invasive 30 potential of budding cancer cells as compared with cancer cells located deeper in the tumor (Hase et al., Dis. Colon Rectum, 1993, 36:627-635). Therefore, as demonstrated in Example 3 below, the instant invention allows for the useful prognostic determination

of success of surgery, means for monitoring progression of tumor budding and subsequent prognosis.

The identification of the role of γ -2 chain allows for the novel use of kalinin/laminin 5 γ -2 chain and its ligand, as diagnostic probes of the tumor cell/basement membrane adhesion interface that is crucial for the invasion of non-malignant tissues, and identifies invasive cells.

Thus the identification of the role of γ -2 chain allows for the novel therapeutic intervention of binding of kalinin/laminin 5 to its ligand, and thereby reducing the tumor cell/basement membrane adhesion that is crucial for the invasion of non-malignant tissues, and method for inhibiting the budding of tumor masses, and a means for determining the level of γ -2 chain expression as a measure of budding activity of a given tumor.

As demonstrated in Example 3 below, the γ -2 chain of kalinin/laminin 5 is preferentially expressed by invasively growing malignant cells in human carcinomas. Furthermore, migrating keratinocytes in wound healing also expressed this gene, pointing to a role of γ -2 chain in epithelial cell migration both in malignant and in nonmalignant pathological conditions. The consistent expression of the γ -2 chain gene in invading cancer cells reflects a functional importance of this molecule in vivo in establishing contacts between the invading malignant cells and a provisional matrix in the immediate surroundings of the cancer cells. The instant invention provides methods for the identification of, and diagnosis of invasive cells and tissues, and for the monitoring of the progress of therapeutic treatments.

In a preferred embodiment of this aspect of the instant invention the nucleic acid probe comprise a specifically hybridizing fragment of the γ -2 chain cDNA nucleic acid sequence. In this embodiment, the nucleic acid sequence comprises all or a specifically hybridizing fragment of an open reading frame of the nucleic acid sequence for the γ -2 chain (FIG. 4) encoding the amino acid sequence of the γ -2 chain (FIG. 4). It will be understood that the term "specifically hybridizing" when used to describe a fragment of nucleic acid encoding a human laminin γ -2 chain gene is intended to mean that, nucleic acid hybridization of such a fragment is stable under high stringency conditions of hybridization and washing as the term "high stringency" would be understood by those having skill in the molecular biological arts.

Further, the instant invention provides for the therapeutic treatment of such invasive tissues by using γ -2 chain or biologically active fragments thereof to interfere with the interactions between aberrant γ -2 chain and surrounding tissues. The instant invention also provides for the intervention of γ -2 chain interaction with surrounding tissues by using specific anti- γ -2 chain antibodies (monoclonal or polyclonal) to inhibit the γ -2 chain biological activity.

The instant disclosure also allows one to ablate the invasive cell phenotypic γ -2 chain expression by using genetic manipulation to "knock-out" the functional expression of the γ -2 chain gene in cancer cells, or to completely "knock-out" the functional γ -2 chain gene in the genome of cancer cells. Such knock-outs can be accomplished by using genetic molecular biological techniques for inserting by homologous recombination into genomic DNA, targeted transposon insertion, or random insertion/deletion mutations in the genomic DNA.

The instant disclosure also allows for the therapeutic treatment of invasive cell phenotype by the inhibition of functional γ -2 chain expression in targeted cells by using anti-sense technology, such methods for anti-sense production, stabilization, delivery, and therapeutic approaches are reviewed in Uhlmann et al., 1990, Chem. Reviews 90:543-584).

Moreover, the present invention is also directed to two important functional aspects of the epithelium specific laminin-5, i.e. cell adhesion and migration. First of all, according to the present invention, the γ 2 subunit chain, as such, does not promote cell adhesion and, secondly, the laminin-5 isoform and its γ 2 chain subunit play a role in the migratory process of cells of epithelial origin. According to the present invention, the migratory function of the γ 2 chain is a characteristic for domain III, as shown with antibody inhibition studies. Furthermore, involvement of the γ 2 chain in cell migration was shown to be related with a *cis*-acting element in *LAMC2* gene, as studied in transgenic mice using promoter-reporter gene constructs.

Thus the instant invention provides for a method of detection, diagnosis, prognosis, monitoring, and therapeutic treatment of invasive cell phenotypes.

The examples below are meant by way of illustration, and are not meant to be limiting as to the scope of the instant disclosure.

EXAMPLE 1

Mutation in the γ -2 Chain Gene LAMC2 is critical in some cases of JEB

A unique scanning strategy using RT-PCR amplification of LAMC2 sequences was devised to detect truncated forms of γ -2 chain gene transcripts (Pulkkinen et al., Nature Genetics, 1994, 6:293-298). The 3.6 kilobase coding sequence of the LAMC2 mRNA, was reverse transcribed and amplified with eight pairs of primers, producing overlapping PCR amplimers designated A-H. The PCR products were then examined by agarose gel electrophoresis, followed by MDE heteroduplex analysis. If bands with altered mobility were detected, the PCR products were sequenced, and compared with normal sequences from unaffected family members or unrelated individuals. Intron/exon borders were identified by PCR analysis of genomic DNA, deduced by comparison with cDNA sequences.

15 *A point mutation produces exon skipping*

When a panel of five unrelated JEB patients were analysed, the primers used to amplify segment C (nt 1046-1537) produced a markedly shortened band of 273 base pairs, as compared with the normal 491 base pairs. No evidence of the normal sized band was noted, suggesting that the patient was homozygous for this allele. Direct sequencing revealed that the shortened product resulted from the deletion of 219 base pairs corresponding to nucleotides 1184-1402 in the cDNA, thus exon 9 was deleted. The remaining nucleotide sequences within this and other PCR products did not reveal any additional mutations upon MDE analysis.

Subsequent examination of the genomic DNA revealed that the sequences for exons 8, 9 and 10 were present, however a homozygous G for A substitution at the 3' acceptor splice site at the junction of intron 8 and exon 9, abolished the obligatory splice site sequence (AG).

Examination of another patient revealed that PCR product F (nt 2248-2777) corresponding to domains I and II of the γ -2 chain, was a band with altered mobility. Sequencing the abnormal product revealed a 20 bp deletion, followed by a single base pair (G) insertion in the coding region corresponding to exon 16. This mutation causes a frameshift which results in a premature stop codon 51 base pairs downstream from the

deletion-insertion, producing a truncated kalinin/laminin 5 γ -2 chain terminating at residue 830.

RT-PCR and MDE analyses

5 RNA isolated from fibroblast cell cultures of JEB patients was used as template for RT-PCR of the LAMC2 mRNA. (Epidermal keratinocytes can also be used). cDNA was prepared from 50 μ g of total RNA in a volume of 100 μ L according to manufacturer's recommendations (BRL), and oligonucleotide primers were synthesized on the basis of the cDNA sequence (FIG. 4; Kallunki et al., 1992, supra.), to generate
10 about 500 base pair products, which spanned the entire coding region.

For PCR amplification, 1 μ L of cDNA was used as template and amplification conditions were 94° C. for 5 min followed by 95° C. for 45 sec, 60° C. for 45 sec and 72° C. for 45 sec for 35 cycles in an OmniGene thermal cycler (Marsh Scientific). Amplification was performed in a total volume of 25 μ L containing 1.5 mM
15 MgCl.sub.2, and 2 U Taq polymerase (Boehringer Mannheim). Aliquots of 5 μ L were analysed on 2% agarose gels and MDE heteroduplex analysis was performed according to the manufacturer's recommendation (AT Biochemicals). Heteroduplexes were visualized by staining with ethidium bromide. If a band of altered mobility was detected in heteroduplex analysis, the PCR product was subcloned into the TA vector
20 (Invitrogen), and sequenced by standard techniques.

DNA isolated either from fibroblast cultures or from specimens obtained from buccal smears, was used as template for amplification of genomic sequences. For amplification of introns 8 and 16, about 500 ng of genomic DNA was used as template and the following oligomer primers were utilized.

25 5' GGCTCACCAAGACTTACACA 3' (SEQ ID NO:1);

5' GAATCACTGAGCAGCTGAAC 3' (SEQ ID NO:2

30 5' CAGTACCAGAACCGAGTTCG 3' (SEQ ID NO:3);

5' CTGGTTACCAGGCTTGAGAG 3' (SEQ ID NO:4);

5' TTACTGCGGAATCTCACAGC 3' (SEQ ID NO:5);

5' TACTGTTCACCCAGGGT 3' (SEQ ID NO:6);

5

5' AAACAAGCCCTCTCACTGGT 3' (SEQ ID NO:7);

5' GCGGAGACTGTGCTGATAAG 3' (SEQ ID NO:8);

10 5' CATACCTCTCTACATGGCAT 3' (SEQ ID NO:9);

5' AGTCTCGCTGAATCTCTCTT 3' (SEQ ID NO:10);

5' TTACAACTAGCATGGTGCCC 3' (SEQ ID NO:11);

15

Amplification conditions were 94° C. for 7 min followed by 95° C. for 1.5 min, 56° C. (intron 8) or 58° C. (intron 16) for 1 min and 72° C. for 1.5 min for 35 cycles in an OmniGene thermal cycler (Marsh Scientific). Amplification was performed in a total volume of 25 µL containing 1.5 mM MgCl.sub.2, and 2 U Taq polymerase (Boehringer Mannheim). The PCR products were subcloned and sequenced as above.

20

Verification of Mutations


The putative mutations detected in the PCR products were verified at the genomic level in both cases. For this purpose, a search for a potential change in restriction endonuclease sites as a result of the mutation was performed.

25

Amplification conditions were 94° C. for 7 min followed by 94° C. for 1 min, 58° C. for 45 sec and 72° C. for 45 sec for 35 cycles in an OmniGene thermal cycler (Marsh Scientific). PCR products were analysed on 2.5% agarose gels.

The methods described allow for the screening of patients for mutations in the γ-2 chain which will correlate with JEB. As demonstrated, the results have identified a homozygous point mutation resulting in oxon skipping, and a heterozygous deletion-insertion mutation. This demonstrating the effective screening for, and identification of,

30


γ-2 chain mutations which correlate with JEB. The methods are thus useful for diagnosis, prenatal screening, early screening and detection, as well as detailed examination of JEB. Further, the results show that the functional role of γ-2 chain expression in epithelial cells is important in determining proper intercellular connectivity, relating to the integrity of tissues and cell interactions.

EXAMPLE 2

Mutation in the γ-2 Chain Gene LAMC2 is Critical in H-JEB

The correlation both in vivo and in vitro of the dermo-epidermal separation in H-JEB, with deficient immunoreactivity of anti-nicein/kalinin/laminin 5 antibodies, and the separation induced by anti-nicein/kalinin/laminin 5 antibodies have made the genes encoding this protein strong candidates for the site of H-JEB mutations. In this example, it is demonstrated that the molecular defect which causes H-JEB is linked to the gene encoding nicein/kalinin/laminin 5 γ-2 chain. In particular, the occurrence of a homozygous premature termination codon mutation is the specific cause in an examined case of H-JEB (Aberdam et al., Nature Genetics, 1994, 6:299-304).

Expression of mRNA encoding the three nicein subunits by northern analysis of RNA isolated from primary keratinocyte culture of a H-JEB patient was determined as the initial screen. Hybridization with probes for the α-3 and β-3 subunits was normal, but no hybridization with a cDNA encoding the γ-2 subunit was detected. Examination of the genomic DNA for gross abnormalities, such as large deletions, insertions or rearrangements, in LAMC2 (the γ-2 subunit gene) by Southern blot analysis turned up no abnormalities when the genomic DNA was digested with BamHI, BglII, HindIII, PstI or PvuII and probed with full length LAMC2 cDNA.

Possible mutations in the γ-2 subunit were sought by using cDNA reverse transcribed from total RNA purified from cultured keratinocytes of the H-JEB patient, and subjected to PCR amplification. The size of the amplified products was checked by electrophoresis on 2% agarose gels and compared with that obtained from healthy controls.

No major differences were detected in the agarose gels, and the PCR products were examined by heteroduplex analysis (MDE). Heteroduplex analysis of the most 5'

PCR product (nt 35-726) revealed the presence of a homoduplex in the proband (pateint) and the controls. However, when the amplified PCR products from the patient and control were mixed together, an additional band with altered mobility, representing heteroduplexes, was detected, suggesting a homozygous mutation in the patient's
5 LAMC2 cDNA. This amplified fragment corresponded to domain V of the γ -2 protein (Vailly et al., Eur. J. Biochem., 1994, 219:209-218). Sequencing detected a C to T transition at position +283, leading to a nonsense mutation in which a termination codon TGA replaces an arginine (CGA), perhaps arising as a result of the hypermutability of 5-methyl-cytosine to thymine at CpG nucleotides. This mutation, R95X, leads to
10 truncation of the γ -2 subunit polypeptide at amino acid 95 and loss of a TaqI restriction site (TCGA). Digestion of cDNA with TaqI confirmed the presence of a homozygous mutation in the DNA of the H-JEB patient. No other mutations were detected.

To confirm the cosegregation of the mutation with the loss of the TaqI restriction site, eight genotyped individuals of the family of the patient were screened. In
15 each case, a 120 base pair fragment was amplified by PCR using genomic DNA templates and primers flanking the restriction site. Upon digestion of the wild-type amplification product, two cleavage fragments of 80 and 40 base pairs are generated. Consistent with the presence of a heterozygous mutation in carriers of this genotype, DNA fragments of 120, 80 and 40 base pairs, indicative of a wild type genotype, were
20 found in the paternal grandmother and two other relatives.

Cell Culture

Epidermis was separated from dermis by dispase treatment at 37 C. Keratinocytes were dissociated in 0.25% trypsin at 37° C. and plated onto a feeder layer
25 of irradiated mouse 3T3 cells (ICN) (Rheinwald & Green, Cell, 175, 6:331-334). Keratinocytes were grown in a 1:1 mixture of DMEM and Ham's F12 (BRL) containing 10% Fetal Calf Serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 μ g/mL of penicillin and strptomycin, 10 ng/mL transferrin, 180 μ M adenine and 20 pM T3 (Simon & Green, Cell, 1985, 40:677-683). H-JEB keratinocytes were expanded after gentle
30 dissociation in 0.05% trypsin, 0.02% EDTA.

Northern Blot Analysis

Total RNA was prepared from H-JEB and normal cultured keratinocytes according to standard methods (Chomzynski & Sacchi, Anal. Biochem., 1987, 162: 156-159). RNA was electrophoresed in 1.2% denaturing agarose gels containing 1.2M formaldehyde and transferred onto Hybond N membrane (Amersham). Membranes were hybridized at high stringency with P-32 labeled cDNA probes corresponding to the different chains of nicein, and then exposed on Hyperfilm MP (Amersham) with intensifying screens. Radiolabeled cDNA probes NA1 (Baudoin et al., J. Invest. Dermatol., 1994, in press), KAL-5.5C (Gerecke et al., Eur. J. Biochem., 1994, in press), and PCR 1.3 (Vailly et al., 1994, supra.), were used to detect the mRNAs for nicein chains α -3, β -3 and γ -2, respectively.

RT-PCR and heteroduplex analysis (MDE)

50 μ g of total RNA isolated from cultured keratinocytes from JEB patient, and unrelated healthy controls were reverse transcribed in a volume of 100 μ L as recommended by the manufacturer (BRL). 1 μ L of the reaction product was used to amplify overlapping regions of the cDNA that spanned the open reading frame. Primer pair used to identify the mutation R95X: (L) 5'-GAGCGCAGAGTGAGAACCAC-3' SEQ ID NO:16, (R) 5'-ACTGTATTCTGCAGAGCTGC-3' SEQ ID NO:17. PCR cycling conditions were: 94° C., 5 min, followed by 94° C., 45 sec; 60° C., 45 sec; 72° C., 45 sec; for 35 cycles, and extension at 72° C. for 5 min. 5 μ L aliquots were run in 2% agarose gels. Heteroduplex analysis was performed as recommended by the manufacturer (MDE, AT Biochemicals). Heteroduplexes were visualized under UV light in the presence of ethidium bromide and photographed. Amplified cDNA fragments with altered mobility were subcloned into the TA vector according to the manufacturer's recommendations (Invitrogen). Sequence analysis were then performed using standard techniques.

Verification of the mutation

PCR reactions on genomic DNA (50 μ g) were carried out using the upstream primer 5'-TTCCTTTCCCCTACCTTGTG-3' (SEQ ID NO:18) and the downstream primer 5'-TGTGGAAGCCTGGCAGACAT-3' (SEQ ID NO:19), which are located in

the intron 2 and exon 3 of LAMC2 respectively. PCR conditions were: 95° C., 5 min, followed by 94° C., 45 sec; 56° C., 45 sec; 72° C., 45 sec; for 35 cycles, and extension at 72° C. for 5 min. PCR products were used for restriction analysis. 20 µL of PCR product obtained from genomic DNA was digested with TaqI for 2 hours (Boehringer Mannheim). Cleavage products were electrophoresed (2.4% agarose) stained and visualized under UV light.

Thus the methods allow for the screening of patients for mutations in the γ -2 chain which correlate with H-JEB. As demonstrated, the results have identified a nonsense mutation resulting in a truncated γ -2 chain, leading to severe H-JEB. This was further confirmed by specific amplification and restriction enzyme analysis of both the patient and relatives. Thus demonstrating the effective screening for, and identification of, γ -2 chain mutations which correlate with H-JEB. The methods are thus useful for diagnosis, prenatal screening, early screening and detection, as well as detailed examination of H-JEB. Furthermore, the results demonstrate the significance of the γ -2 chain in forming proper cellular contacts.

EXAMPLE 3

γ -2 Chain as Diagnostic for Invasive

In this example, in situ hybridization is used to demonstrate the expression of the kalinin/laminin 5 γ -2 chain in a variety of human cancer tissues and in skin wound healing in mice (Pyke et al., Amer. J. Pathol., Oct. 1994, 145(4):1-10).

Thirty-six routinely processed, formalin-fixed and paraffin wax-embedded specimens from cancer surgery performed from 1991 to 1993 were drawn from pathology department files at Herlev Hospital (Copenhagen, Denmark). The specimens were evaluated according to standard criteria and included 16 cases of moderately or well-differentiated colon adenocarcinomas, 7 cases of ductal mammary carcinomas, 4 squamous cell carcinomas (2 skin, 1 cervix, 1 vulva), 3 malignant melanomas, and 6 sarcomas (3 leiomyosarcomas, 2 malignant fibrous histiocytomas, 1 neurofibrosarcoma).

All samples were selected upon histological examination of a hematoxylin and eosinstained section to ensure that they showed a well preserved morphology throughout and contained representative areas of both cancerous tissue and surrounding apparently

normal, unaffected tissue. The broad zone separating these two tissue compartments is referred to as the invasive front in the following. No estimation of the effect of variations in fixation conditions was attempted, but in a previous study of plasminogen activating system components using specimens of colon adenocarcinomas collected using the same procedures, very little variation in relative mRNA levels was found (Pyke, C. PhD. Thesis, 1993, University of Copenhagen, Denmark). In addition, tissue from incisionally wounded mouse skin prepared as described by Romer et al. (J. Invest. Dermatol., 1994, 102:519-522), was fixed and paraffin-embedded the same way as the human cancer specimens.

For preparation of total RNA from six samples of colon adenocarcinomas, tissues were snap-frozen in liquid nitrogen immediately following resection and RNA was prepared as described by Lund et al., (Biochem. J., 1994).

Probes:

Fragments of the cDNA for the γ -2 chain of human kalinin/laminin 5 were inserted into RNA transcription vectors by restriction enzyme cutting of clone L15 covering base pairs 2995 to 3840 (FIG. 4; Kallunki et al., 1992, supra.). In brief, plasmids phb2t-01 and phb2t-02 were prepared by insertion of the complete L15 γ -2 chain cDNA in sense and anti-sense orientation into the polylinker of plasmid vectors SP64 and SP65 (both Promega, Madison, Wis.), respectively. In addition, two non-overlapping fragments of clone L15 were bluntend cloned into the EcoRV-site of pKS(Bluescript)II(+) (Stratagene, La Jolla, Calif.) transcription vector and the resulting plasmids were verified by dideoxy sequencing according to Sanger et al. (PNAS(USA), 1977, 74:5463-5471). Plasmid phb2t-03 cover bases 3003-3239 and phb2t-05 cover bases 3239 to 3839, numbers referring to cDNA sequence Z15008 in the EMBL/GenBank/DDBJ database as reported by Kallunki et al., (1992, supra.; FIG. 4).

Similarly, cDNA fragments of other human laminin chains were prepared in RNA transcription vectors, yielding the following plasmid constructs (numbers in brackets refer to base pair numbers in the EMBL/GenBank/DDBJ sequence database by the listed accession numbers); chain α -1: plasmid phae-01 (3244-3584 (accession No. X58531, Nissinen et al., Biochem. J., 1991, 276:369-379) in pKS(Bluescript)II(+)); chain β -1: plasmid phb1e-01 (3460-4366 (accession No. J02778, Pikkarainen et al., J.

Biol. Chem., 1987, 262:10454-10462) in pKS(Bluescript)II(+)); chain γ -1: plasmids A1PSP64 and A1PSP65 (919-1535 (accession No. M55210, Pikkarainen et al., J. Biol. Chem., 1988, 263:6751-6758) in SP64 and SP65 respectively (sense and anti-sense orientation)).

5 All plasmids were linearized for transcription using restriction endonucleases and 5 μ g of the linearized plasmids was extracted with phenol and with chloroform/isoamyl alcohol (25:1), precipitated with ethanol, and redissolved in water. Each transcription reaction contained 1 μ g linearized DNA template, and transcriptions were performed essentially as recommended by the manufacturer of the polymerases.
10 The RNA was hydrolyzed in 0.1 mol/L sodium carbonate buffer, pH 10.2, containing 10 mmol/L dithiothreitol (DTT) to an average size of 100 bases. RNA probes transcribed from opposite strands of the same plasmid template, yielding sense and anti-sense transcripts, were adjusted to $\times 10^{5.6}$ cpm/ μ L and stored at -20° C. until used. Probes were applied to tissue sections.

15

In situ Hybridization:

In situ hybridization was performed as described by Pyke et al., (Am. J. Pathol., 1991, 38:1059-1067) with 35S labeled RNA probes prepared as described above. In brief, paraffin sections were cut, placed on gelatinized slides, heated to 60° C.
20 for 30 minutes, deparaffinized in xylene, and rehydrated through graded alcohols to PBS (0.01 mol/L sodium phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl). The slides were then washed twice in PBS, incubated with 5 μ g/mL proteinase K in 50 mmol/L Tris/HCl, pH 8.0, with 5 mmol/L EDTA for 7.5 minutes, washed in PBS (2 minutes), dehydrated in graded ethanols, and air-dried before the RNA probe (about 80 pg/ μ L)
25 was applied. The hybridization solution consisted of deionized formamide (50%), dextran sulfate (10%), tRNA (1 μ g/ μ L), Ficoll 400 (0.02% (w/v)), polyvinylpyrrolidone (0.02% (w/v)), BSA fraction V (0.02% (w/v)), 10 mmol/L DTT, 0.3M NaCl, 0.5 mmol/L EDTA, 10 mmol/L Tris-HCl, and 10 mmol/L NaPO₄ (pH 6.8). Sections were covered by alcohol-washed, autoclaved coverslips and hybridized at 47° C.
30 overnight (16 to 18 hours) in a chamber humidified with 10 ml of a mixture similar to the hybridization solution, except for the omission of probe, dextran sulfate, DTT, and tRNA (washing mixture). After hybridization, slides were washed in washing mixture

for 2 x 1 hour at 50° C., followed by 0.5 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 7.2) (NTE) with 10 mmol/L DTT at 37° C. for 15 minutes. After treatment with RNase A (20 µg/mL) in NTE at 37° C. for 30 minutes, the sections were washed in NTE at 37° C. (2 x 30 minutes), and in 2 L of 15 mmol/L sodium chloride, 1.5 mmol/L sodium citrate, pH 7.0, with 1 mmol/L DTT for 30 minutes at room temperature with stirring. Sections were then dehydrated and air-dried. Finally, autoradiographic emulsion sec applied according to the manufacturer's recommendations, and sections were stored in black airtight boxes at 4 C. until they were developed after 1 to 2 weeks of exposure.

10 *Results; Laminin α -1, β -1, γ -1, and γ -2 chains*

All rounds of in situ hybridization include both sense and anti-sense RNA probes for each of the genes studied. As negative controls, sense RNA probes are applied to adjacent sections and these probes consistently are negative. As a positive control of the γ -2 chain hybridizations, two anti-sense probes derived from non-overlapping γ -2 chain cDNA clones are used on a number of sections. To summarize the γ -2 chain expression found; all carcinomas were positive except for one case of mammary duct carcinoma, and all three cases of leiomyosarcomas, both cases of malignant fibrous histiocytoma, and the only case of neurofibrosarcoma. The positive controls always give similar staining on adjacent sections (see FIG. 2, E and G). Fifteen of the malignant cases and all mouse tissue blocks were hybridized on two or more separate occasions giving the same hybridization pattern. All cell types other than those described below were negative in all cases.

Colon Adenocarcinoma

25 Sixteen specimens of colon adenocarcinoma were investigated by in situ hybridization for expression of the γ -2 chain (FIG. 1). In all of these cases, mRNA for γ -2 chain was present exclusively in cancer cells and in most of the cases, staining was confined to a distinct subpopulation of cancer cells at the invasive front (FIG. 1, A-D). A characteristic feature of γ -2 chain containing cancer cells at the invasive front was that they appeared to represent cells in the process of branching or dissociating from larger well differentiated epithelial glands, a phenomenon referred to in the literature as tumor budding or tumor-cell dissociation.

In normal-looking colon mucosa distal from the invasive carcinoma, moderate signals for γ -2 chain mRNA were observed in two specimens in the epithelial cells of a few mucosal glands that showed clear morphological signs of glandular disintegration and phagocytic cell infiltration. Apart from this, a weak signal was seen in luminal epithelial cells in normal looking colon mucosa in most specimens.

Weak signals for laminin chains α -1, β -1, and γ -1 mRNAs were detected in cancerous areas of the 6 colon cancers studied for the expression of these genes. The expression of each of the three genes showed a similar distribution. Expression in stromal cells with a fibroblast-like morphology as well as in endothelial cells of smaller vessels was consistently found. In marked contrast to the γ -2 chain expression in the same samples, expression of α -1, β -1, or γ -1 was never found in cancer cells and no correlation between expression of α -1, β -1, and γ -1 chains with sites of invasion was found. Adjacent normal-looking parts of the samples were negative or only weakly positive for these laminin chains.

FIG. 1 shows in situ hybridization of a specimen of colon adenocarcinoma for γ -2 chain mRNA using a S-35 labeled anti-sense RNA probe derived from plasmid pbb2r-02. FIG. 1A is a cluster of heavily labeled cancer cells at the invasive front (open arrow) in close proximity to a well-differentiated glandular structure (straight arrow). FIG. 1B shows a high-magnification view of the area at the open arrow in 1A. Note that the isolated cancer cells show prominent labeling, whereas many coherent cancer cells of an adjacent glandular structure are negative (straight arrow). FIG. 1C shows the same pattern at an invasive focus in another part of the same specimen. FIG. 1D shows strong γ -2 chain expression in cancer cells engaged in a bifurcation process (curved arrows). The malignant glandular epithelium from which the γ -2 chain-positive cancer cells are branching is negative (straight arrow). Magnification: 1A \times 100; 1B-1D \times 640.

Ductal Mammary Carcinomas

Six of the seven cases showed a prominent signal for γ -2 chain in a small subpopulation of cells intimately associated with invasively growing malignant glandular structures. The most prominent signal was seen in cells located at the border between malignant and surrounding stromal tissue in glandular structures that exhibited clear histological signs of active invasion (FIG. 2A). On careful examination it was

concluded that the majority of the positive cells were cancer cells but it was not possible to determine if the cells of myoepithelial origin were also positive in some cases. One case was totally negative. Normal-appearing glandular tissue was negative in all cases.

Weak signals for laminin chains α -1, β -1, and γ -1 mRNAs were detected in
5 fibroblast-like stromal cells throughout cancerous areas in one of the cases.

Malignant Melanoma

In all three cases strong hybridization of γ -2 chain was found in a population of cancer cells in the radial growth phase (FIG. 2B). Laminin chains α -1, β -1, and γ -1
10 were weakly expressed in the endothelium of small vessels and in fibroblast-like stromal cells throughout the affected areas in the two cases studied for these components. In addition, a weak signal for these chains was seen in sebaceous glands of adjacent normal skin.

Squamous Cell Carcinomas

In all four squamous cell carcinomas investigated, the same pattern of γ -2 chain expression was found as in other carcinomas. The signals were found only in cancer cells, and only in areas with signs of ongoing invasion (FIG. 2, C-G).

The four cases were also studied for mRNA of α -1, β -1, and γ -1 chains. In
20 the two skin cancers, it was found that a very weak signal occurred in malignant cells, and that the weak signal was in all cancer cells and of an equal intensity. This is in clear contrast to the pattern of expression of the γ -2 chain. As seen in melanomas, epithelial cells of sebaceous glands present in adjacent unaffected skin were weakly positive for these laminin chains. In the other two cases (cervix and vulva) weak expression of α -1,
25 β -1, and γ -1 chains were seen only in endothelial and fibroblast-like stromal cells throughout the cancerous areas (FIG. 2F).

FIG. 2 shows In situ hybridization for γ -2 chain mRNA on sections of ductal mammary carcinoma (2A), malignant melanoma (2B), squamous cell carcinoma of the skin (2C-2D), and squamous cell carcinoma of the vulva (2E-2G). In 2A, cancer shows
30 prominent signal for γ -2 chain mRNA in cells bordering the zone between malignant glandular tissue and surrounding mesenchyme (curved arrows). Cancer cells located more centrally in individual malignant glandular structures are negative for γ -2 chain

mRNA (straight arrows). Note the wedge shaped form of the invading glandular tissue. (All images marked X' are darkfield images of the respective sections). FIG. 2B shows γ -2 chain mRNA signal in a subpopulation of cancer cells of radially growing malignant epithelium (curved arrows). Adjacent malignant epithelium showing a different growth pattern is devoid a signal (straight arrow). FIG. 2C shows γ -2 chain mRNA containing cancer cells at the invasive front (curved arrow). Note lack of signal in non-invasive areas of the tumor and in adjacent unaffected areas (straight arrow). FIG. 2D is a higher magnification of area of curved arrow of 2C highlighting the prominent signal in invading cells (curved arrow). Adjacent cancer cells with tumor islets are negative (straight arrow). FIG. 2E shows a strong signal for γ -2 chain mRNA is seen in invading cancer cells, using an anti-sense RNA probe derived from plasmid pb2t-03 (curved arrow). A postcapillary venule is negative (straight arrow). FIG. 2F is a near adjacent section hybridized for laminin γ -1 chain. Note that the endothelial cells of the venule show signal (straight arrow) whereas the malignant epithelium is negative (curved arrow). FIG. 2G is another near-adjacent section which was hybridized for γ -2 chain expression using an anti-sense RNA probe derived from a cDNA plasmid non-overlapping with that used for preparing the probe in 2E (phb2t-05). Note that the hybridization pattern is similar to that seen in 2E, with strong signal in invading cancer cells (curved arrow) and absence of signal in a vessel (straight arrow). Magnification: 2C \times 100, all others \times 640.

Sarcomas

All six sarcomas tested in the study were totally negative for γ -2 chain mRNA. The expression of other laminin chains was not tested.

Mouse Wounded Skin

To compare the gene expression of γ -2 chain in cancer tissue with a nonmalignant condition known to contain actively migrating epithelial cells showing a transient invasive phenotype, we hybridized sections of incisionally wounded mouse skin with γ -2 chain sense and anti-sense RNA probes. Weak γ -2 chain expression was observed in the keratinocytes at the edge of 12-hour old wounds, and at later time points (1-5 days), strong signals for γ -2 chain mRNA was seen exclusively in the basal

keratinocytes of the epidermal tongue moving under the wound clot (FIG. 3). In adjacent normal-looking skin, keratinocytes were negative for γ -2 chain mRNA.

FIG. 3 is incisionally wounded mouse skin (γ -2 hours after wounding) showing signal for γ -2 chain in keratinocytes at the leading edge of the migrating epithelium (curved arrow). Whereas buccal keratinocytes located more distant to the site of injury show little or no signal (straight arrow). Note that the signal for γ -2 chain stops at the tip of invading keratinocytes (open arrow). A' is a dark field image of 3A. Magnification: \times 640.

RNAse Protection Assay

Plasmid phbt-03 was linearized with EcoRI and a radiolabeled RNA-anti-sense probe was prepared by transcription using γ -sup.32 P UTP and T3 polymerase (Pyke et al., FEBS Letters, 1993, 326:69-75). RNAse protection assay, using 40 μ g ethanol-precipitated and DNase μ I-treated total RNA from six samples of colon adenocarcinomas was performed as described in Pyke et al., (1993, supra.). Protected mRNA regions were analyzed on a denaturing polyacrylamide gel and autoradiography.

The RNAse protection assay carried out on total RNA from the six samples confirmed the presence of genuine γ -2 chain mRNA in all samples.

These results clearly demonstrate the important correlation of γ -2 chain expression and invasive cell phenotype in vivo, as detected in vitro. Thus the instant methods present a novel and important method for the specific identification of invasive cell phenotypes in biopsied tissues. The knowledge of any information diagnostic for the presence or absence of invasive cells is useful for the monitoring and prognosis of continuing anti-carcinoma therapies. Further the identification of the expression or non-expression of the γ -2 chain provides important information as to the phenotypic nature of the tissue examined. Thus the instant example demonstrates the use of probes of γ -2 chain for detection of the presence, or absence, of invasive cells.

EXAMPLE 4

The following example demonstrates the functional aspects of laminin-5, including the γ -2 chain of laminin-5, on cell adhesion and cell migration.

Materials and Methods

Cells and Cell Culture - A mouse squamous cell carcinoma cell line, KLN-205 (cat. no. ATCC CRL-1453), was obtained from American Type Culture Collection (Rockville, MD). The cells were maintained as monolayer cultures in Eagle's minimum essential medium (MEM) containing non-essential amino acids and Earle's BSS supplemented with 10 % fetal calf serum (FCS). The HaCat human keratinocyte cell line was a kind gift from Dr. Fuzenig (Heidelberg, Germany). The HaCat cells were cultured in Dulbecco's MEM supplemented with 10 % FCS. However, when the cells were cultured for the production of laminin-5, the medium was replaced by serum-free medium.

Preparation of Proteins - Mouse EHS laminin (laminin-1) was obtained from GIBCO BRL. Fibronectin was purified from FCS using a gelatin-Sepharose 4B column (Sigma) as described elsewhere (Vuento, M. & Vaheri, A. (1979) Biochem. J. 183: 331-337.34. Gillies, R. J., Didier, N. & Denton, M. (1986) Anal. Biochem. 159: 109-113). Human laminin-5 was immunoaffinity purified from the media of HaCat cells cultured for three days in the absence of serum. Briefly, the medium was first passed through a 5 ml gelatin-Sepharose column (Sigma, St. Louis, MO) to ensure the complete absence of fibronectin from the protein preparation, after which the medium was passed through a 10 ml anti-laminin γ 2-Sepharose affinity column in order to bind laminin-5 molecules. Both columns were equilibrated in phosphate-buffered saline. The anti-laminin γ 2-Sepharose affinity column was prepared by coupling a Protein A-purified anti- γ 2 IgG (8 mg/ml) to 10 ml of CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden). The anti- γ 2 IgG was purified from a rabbit polyclonal antiserum prepared against a GST-fusion protein containing part of the short arm (domain III) of the γ 2 chain (Pyke, C., Salo, S., Ralfkiaer, E., Romer, J., Dano, K. & Tryggvason, K. (1995) Cancer Res. 55: 4132-4139). The laminin-5 was eluted from the immunoaffinity column using 50 mM triethanolamine, pH 11.25, 0.1 % Triton X-100 and neutralized directly with 1 M Tris-HCl, pH 7.0. Collected fractions were analyzed by SDS-PAGE and Western blotting using the same polyclonal antibodies as used for the preparation of the affinity column. Fractions containing laminin-5 were pooled and dialyzed against 50 mM Tris-HCl, 0.1 M NaCl, pH 7.4. Some batches of laminin-5 were denatured with 5 M urea and renatured to study the effects of the treatment on adhesion and migration properties.

Generation of Recombinant Baculovirus and Expression of Recombinant Laminin γ 2 Chain

The γ 2 chain of laminin-5 was expressed as recombinant protein using the baculovirus system and purified for studies on its functional properties. A full-length human laminin γ 2 chain cDNA containing 6 bp of the 5' UTR and 822 bp of the 3' UTR was constructed from four overlapping cDNA clones L52, HT2-7, L15 and L61 (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) J. Cell Biol. 119: 679-693). The resulting 4,402 bp cDNA was analyzed by restriction enzyme mapping and partial sequencing, and cloned into the pVL1393 recombinant transfer plasmid prior to transfer into the AcNPV- γ 2 baculovirus vector kindly provided by Max Summers (Texas A&M University). This baculovirus vector containing the human laminin γ 2 chain cDNA under the transcriptional control of the polyhedrin promoter was produced and purified following standard procedures (Summers, M. D. & Smith, G. E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. Texas agricultural experiment station bulletin no. 1555, Collage Station, Texas), except that it was first enriched according to the method of Pen et al. (Pen, J., Welling, G.W. & Welling-Wester, S. (1989). Nucl. Acid. Res. 17: 451) from the virus containing medium obtained by co-transfecting Sf9 cells with the wild-type virus (AcNPV) DNA and the recombinant transfer vector pVL1393- γ 2. For expression of the recombinant protein, High Five (H5) cells were infected with the recombinant virus at a multiplicity of infection (MOI) of 5 - 10 pfu per cell by using the standard protocols (Summers, M. D. & Smith, G. E. (1987)).

The recombinant γ 2 chain was purified by first resuspending the cells in 10 volumes of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 1 % Triton X-100, 1 mM PMSF and 1 mM NEM followed by homogenization in a Dounce homogenizer. The protein was extracted for 60 min on ice and solubilized proteins were removed by centrifugation at 1500 x g for 10 min at 4 °C. The pellet was extracted again with buffer containing 1-3 M urea. The recombinant γ 2 chain was extracted with a buffer containing 5 M urea, and renatured by dialysis against 50 mM Tris-HCl, pH 7.4, 100 mM NaCl.

Preparation of Antibodies - Polyclonal antiserum against domain III of the laminin $\gamma 2$ chain was prepared and characterized as described previously. Briefly, rabbits were immunized s.c. four times using a $\gamma 2$ -GST fusion protein as antigen. The antigen contained 177 amino acid residues (res. # 391-567) from domain III of the $\gamma 2$ (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) J. Cell Biol. 119: 679-693). Antibodies against the GST-epitopes were removed from the antisera by negative immunoadsorption with GST-Sepharose made by coupling *E. coli* expressed GST protein to CNBr-activated Sepharore. The removal of anti-GST IgG was ensured by Western blotting analysis with GST-specific antibodies (data not shown). The specificity of the antibody against the laminin $\gamma 2$ chain was also tested by Western blotting as well as by ELISA.

Polyclonal antibody against the C-terminus of the laminin $\gamma 2$ chain was produced in rabbits essentially as above for domain III using a $\gamma 2$ -GST fusion protein as antigen. The antigen contained 161 amino acids (res. # 1017-1178) from domain I/II of the $\gamma 2$ chain and antibodies against the GST-epitopes were removed from the antisera by negative immunoadsorption with GST-Sepharose. The specificity of the antibody was tested by Western blotting and ELISA.

Polyclonal antiserum against laminin-1 was a kind gift of Dr. Foidart (University of Liege, Belgium). Normal rabbit serum was obtained prior to immunization from the rabbits used for immunization. IgG from the laminin-1 and laminin $\gamma 2$ chain antisera, as well as from normal rabbit serum, was purified using Protein A Sepharose (Pharmacia, Uppsala, Sweden).

Cell Adhesion Assay - Microtiter plates (96 wells: Nunc, Copenhagen, Denmark) were coated with 100 μ l/well of laminin-1 (10 μ g/ml), laminin-5 (10 μ g/ml), or recombinant laminin $\gamma 2$ chain (10 μ g/ml) in PBS or 50 mM Tris-HCl, pH 7.4 by incubating the plates overnight at 4° C. Control wells were uncoated or coated with the same amounts of BSA. In some experiment the proteins were first denatured by dialysis overnight against 5 M urea, 50 mM Tris-HCl, pH 7.5 and then renatured by dialysis against 50 mM Tris-HCl, pH 7.5. Potential remaining active sites on the plates were blocked with 150 μ l of 10 mg/ml BSA in PBS for 2 hours at room temperature. The wells were washed with PBS, and 100 ml of Eagle's MEM containing 5 mg/ml BSA

was added. For the adhesion assays, KLN-205 cells were detached from subconfluent cell culture dishes with trypsin-EDTA (0.25%-0.03%) and resuspended in Eagle's MEM/BSA (5 mg/ml) at a concentration of 2×10^5 cells/ml and allowed to recover for 20 min at 37° C. A total of 20,000 cells were then added to each well and allowed to attach for additional 90 min at 37° C. The extent of cell adhesion was determined by measuring color yields at 600 nm, following fixation with 3 % paraformaldehyde and staining with 0.1 % crystal violet (Gillies, R. J., Didier, N. & Denton, M. (1986) Anal. Biochem. 159: 109-113). For inhibition assays with the anti- $\gamma 2$ antibody, the substrate coated wells were incubated with 20 μ g/ml of anti- $\gamma 2$ chain IgG in PBS for 60 minutes prior to incubations with the cells.

Migration assay - The effect of endogenous laminin-5 on migration of KLN-205 cells was determined by using a modified Boyden chamber assay, as described by Hujanen and Terranova (Hujanen, E. & Terranova, V.P. (1985) Cancer Res. 45: 3517-3521), and the effect of exogenous laminin-5 by using a modified Transwell assay, as described by Pelletier et al. (Pelletier, A. J., Kunicki, T. and Quaranta, V. (1996). J Biol Chem 271: 1364).

The Boyden chamber assay was briefly carried out as follows. Polycarbonate filters (pore size 10 μ m, diameter 12 mm; Costar, Cambridge, MA) were coated with 2.5 μ g of EHS type IV collagen, and used to separate the upper and lower compartments of the 50 μ l chamber. A total of 1×10^5 cells in Eagle's MEM containing 0.1 % BSA were placed in the upper compartment, and the lower compartment was filled with medium with or without chemoattractants (50 μ g/ml laminin-1 or fibronectin). To study the effect of the laminin $\gamma 2$ chain antibodies on cell migration, anti- $\gamma 2$ (III) IgG or anti- $\gamma 2$ (C-term) IgG was added to the upper compartment together with the cells at a concentration 20 μ g/ml. Normal rabbit IgG was used as negative control. After 8 hour incubation at 37°C in a humidified atmosphere, the filters were removed, fixed and stained (Diff-Quick, Baxter Diagnostics, Tubingen, Germany). The cells that had not migrated were removed from the upper surface of the filter with cotton swabs. Migration of cells was quantified by counting the cells on the lower surface of each filter in 10 randomly selected high power fields (x400). All assays were performed in triplicates.

The "Transwell" plate assay (Transwell plates with pore size 12 μ m, diameter 12 mm; Costar, Cambridge, MA) was used to determine the effect of exogenous laminin-5 on cell migration. The lower side of the membrane was coated with 2.5 μ g of EHS type IV collagen for 3 hours at room temperature. Both side were blocked with 1 % bovine serum albumin for 1 hour. A total of 1×10^5 cells were added per well in the upper compartment in Eagle's MEM containing 10 % FCS, and the lower compartment was filled with 2.5 μ g/ml laminin-5 as a chemoattractant. Antibodies against the C-terminus and domain III of the γ 2 chains or nonimmune IgG were added to the upper compartment, together with the cells at a concentration 20 μ g/ml. Following a 16 hour incubation at 37° C the cells were fixed and stained with Diff-Quick. Cells on the top surface of the membrane were removed with cotton swabs, and cells that had migrated to the lower side of the membrane were counted (12 fields +/- S.D.)

Preparation of LAMC2-lacZ Reporter Gene Constructs - Two different segments of the LAMC2 5' flanking regions were subcloned into a pKK2480 vector containing the β -galactosidase gene and the SV40 polyadenylation signal (kind gift from Mikkel Rohde, Aarhus, Denmark) for expression in transgenic mice. The longer construct, pHH-1, contained 5946 base pairs (-5,900 to +46) and the shorter construct contained a total of 668 base pairs (-613 to +55). Briefly, the 5' end of the pHH-1 insert was made by ligating a 3,900 base pair HindIII-PstI fragment and a 1,150 base pair PstI fragment of genomic clone P14 (Airenne, T., Haakana, H., Sainio, K., Kallunki, T., Kallunki, P., Sariola, H. & Tryggvason, K. (1996) Genomics 32: 54-64). The 3' end of the construct was a PstI-SalI fragment (-699 to +46) made by PCR and ligated to the 5' end 5,050 base pair fragment. The full length fragment was blunt ended with Klenow and subcloned into the pKK2480 vector. The shorter construct pHH-2 was made by PCR from genomic clone P14, digested with SalI and XbaI and subcloned into pKK2480. All PCR made segments were sequenced to ensure that no sequence errors existed. Both constructs could be released from the vector with XhoI and EagI.

Generation and Analyses of Transgenic Mice - The plasmids pHH-1 and pHH-2 were digested with EagI and XhoI to release the inserts from the vector. Transgenic mice were produced by pronuclear microinjection of (C57Bl/6+DBA/2)F1 fertilized oocytes as

described elsewhere (Hogan, B., Constantini, F. & Lacy, E. (1986) Manipulating the mouse embryo: A Laboratory Manual. Cold Spring Harbor, New York). Founder animals were identified by PCR analysis (Hanley, T. & Merlie, J. P. (1991) BioTechniques 10: 56) or Southern blotting of genomic DNA isolated from the tail.

5 Positive founder mice were mated with wild-type hybrid (C57Bl/6+DBA/2)F1 mice to yield transgenic lines. Expression of the *lacZ* gene was detected by staining with X-gal (5bromo-4-chloro-3-indolyl-b-D-galactopyranoside) as a substrate (Behringer, R. R., Crotty, D. A., Tennyson, V. M., Brinster, R. L., Palmiter, R. D. & Wolgemuth, D. J. (1993) Development 117: 823-833).

10

Preparation of Tissues for Immunostaining and Staining for β -Galactosidase Activity -

For immunohistochemical analyses, mouse tissues were fixed in 4 % paraformaldehyde and embedded in paraffin. For analyses of β -galactosidase expression, whole embryos and postnatal tissues were fixed in 0.2 % glutaraldehyde, 2 % paraformaldehyde in 0.1

15 M phosphate buffer, pH 7.3, for 60 min at 4° C washed three times for 30 min with a 7.3 pH 0.1 M phosphate buffer containing 0.1 % sodium deoxycholate, 2 mM MgCl₂ and then stained with X-Gal (1 mg/ml X-Gal, 5 mM K-ferricyanide, 5mM K-ferrocyanide) before embedding in paraffin. Experimental wounds were made to transgenic mice by small cutaneous incisions, the wounds were closed by a single suture, and the wounds

20 surrounded by normal skin were removed surgically after three and seven days and processed for staining.

Immunohistochemical Staining - Five μ m thick paraffin sections were stained with polyclonal antibodies against laminin-1 or the γ 2 chain of laminin-5. In brief, the

25 paraffin sections were first incubated with 0.4 % pepsin in 0.1 M HCl at 37° C for 20 min to expose the antigens, blocked for nonspecific binding with 5 % newborn rabbit serum, 0.1 % BSA, and then incubated for 1 h at 37° C with the polyclonal IgG diluted in TBS to 5 - 10 μ g/ml. Subsequently, a biotinylated swine-anti-rabbit antibody was applied, followed by incubation with a 1:400 dilution of Horseradish-Peroxidase-

30 Avidin-Biotin-Complex (DAKO, Copenhagen, Denmark). The color was developed in diaminobenzamide (DAB), followed by counterstaining of the slides with hematoxylin.

Results

Characterization of Proteins and Epithelium-Derived Cells - Immunopurified trimeric laminin-5, isolated from the culture medium of HaCat cells contained two major bands when analyzed by SDS-PAGE (Fig.1). These bands corresponded, respectively, to the 165 kDa $\alpha 3$ chain, and the 155 kDa and 140 kDa $\gamma 2$ and $\beta 3$ chains migrating as a single band, as reported previously (Carter, W.G., Ryan, M.C. & Gahr, P.J. (1991) Cell 65: 599-610; Rousselle, P., Lunstrum, G.P., Keene, D.R. & Burgeson, R.E. (1991) J. Cell Biol. 114: 567-576; Pikkarainen, T., Schulthess, T., Engel, J. and Tryggvason, K. (1992) Eur. J. Biochem. 209, 571-582). Additionally, a weak band of about 105 kDa corresponding to the processed $\gamma 2$ chain could be observed.

Full-length human recombinant laminin $\gamma 2$ chain was produced in High-5 *Spodoptera frugiperda* insect cells using the baculovirus system. Since the $\gamma 2$ chain was not secreted to the culture medium, possibly because it was not assembled intracellularly into a normal heterotrimer, it was isolated from the cell fraction as described in *Materials and Methods*. The protein was extracted under denaturing conditions using 5 M urea, renatured by extensive dialysis against 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, and purified. The purified recombinant $\gamma 2$ chain was full length and highly pure as determined by SDS-PAGE analysis (Fig. 5).

The HaCat human keratinocytes and mouse KLN-205 squamous carcinoma cells were shown to express laminin-5, based on northern analyses and immunostaining, using a cDNA probe (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) J. Cell Biol. 119: 679-693) and/or polyclonal antibodies specific for the $\gamma 2$ chain (Pyke, C., Salo, S., Ralfkiaer, E., Romer, J., Dano, K. & Tryggvason, K. (1995) Cancer Res. 55: 4132-4139), respectively. Furthermore, the KLN-205 cells developed $\gamma 2$ chain positive primary tumors and metastases in mice *in vivo* (data not shown). Following intramuscular or subcutaneous inoculations, large primary tumors developed in 4 weeks with numerous lung metastases after 4-6 weeks. KLN-205 cells injected into the tail vein produced multiple lung tumors (experimental metastases) in four weeks. Consequently, both cell types were considered appropriate for the cell attachment and migration experiments carried out in this study.

Laminin-5 Molecule, but not Recombinant Laminin γ 2 Chain, Promotes Cell Adhesion -

The laminin-5 and recombinant γ 2 chain prepared in this study, as well as commercial laminin-1, were used as substrata in attachment assays (Fig. 6) with the two epithelium-
5 derived HaCat and KLN-205 cell lines that both express laminin-5. Both cell lines attached about 2.5 times more readily to laminin-1 than to plastic. Adhesion of the cells to laminin-5 appeared to be slightly higher than that to laminin-1, but the differences were not statistically significant. The cells attached equally well to laminin-5 preparations denatured in 5 M urea and then renatured by dialysis against 50 mM Tris-
10 HCl, 100 mM NaCl, pH 7.4, as described for the recombinant γ 2 chain above, indicating that this treatment did not affect the binding properties of the trimeric molecule. The attachment to laminin-5 was not significantly decreased in the presence of two different polyclonal antibodies made against the short or long arms of the γ 2 chain or pre-IgG. Different amounts of the antibody against the short arm of the γ 2 chain were also tested
15 (up to 50 μ g/ml), but no effects on cell adhesion were observed. When the cells were plated on the recombinant γ 2 chain alone, the attachment was not significantly higher than that to plastic, this attachment not being influenced by polyclonal antibodies against the γ 2 chain. The data confirm previous results showing that trimeric laminin-5 promotes adhesion of epithelial cells, but the present results further strongly suggest that
20 this adhesion is not mediated by the γ 2 chain.

Antibodies Against Laminin γ 2 Domain III, but not Domain I/II, Inhibit Cell Migration -

Immunohistochemical and *in situ* hybridization studies on healing skin wounds have indicated a role for laminin-5 in cell migration. The potential role of the γ 2 chain of
25 laminin-5 in this process was examined for the KLN-205 cells *in vitro* using Boyden and Transwell chamber assays as described in *Materials and Methods*.

Migration was first studied in the Boyden chamber assay using laminin-1 and fibronectin in the lower chamber as chemoattractants. The cells were plated on type IV collagen coated filters separating the upper and lower compartments, and antibodies to
30 different domains of the γ 2 chain were added to the upper chamber. Migration of cells in the presence of preimmune IgG was arbitrarily set as 100 % (Fig. 7). When polyclonal IgG against the short arm of the γ 2 chain was added to the upper compartment

containing the cells, the migration of cells through the filter was decreased to about 35 to 45 % of that observed with the preimmune IgG (Fig. 7A). In contrast, the polyclonal IgG against C-terminal domain I/II did not affect migration of the cells (Fig. 7A).

The effects of the two antibodies were similarly used in the Transwell assay using native laminin-5 as chemoattractant in the lower compartment, and the results were essentially the same as above. Thus, addition of IgG raised against domain III of the $\gamma 2$ chain inhibited the migration to about 50 % as compared with preimmune IgG, while the polyclonal IgG against domain I/II did not affect the cell migration.

These *in vitro* results demonstrate that laminin-5 can have a role in the locomotion of epithelium-derived cells, and that this function can be inhibited by antibodies directed against domain III of the $\gamma 2$ chain.

Limited Expression of LAMC2 Promoter-Reporter Gene Constructs in Epithelial Cells in Transgenic Mice - In order to search for potential epithelium-specific enhancer elements in the *LAMC2* gene, we made transgenic mice harboring DNA constructs containing varying lengths of the 5' flanking region of the *LAMC2* gene connected with a downstream reporter gene, which in this case was *LacZ* coding for bacterial β -galactosidase (SEQ. ID. NO.: 20)(Fig. 8). Two different constructs were used for microinjection into pronuclei of fertilized mouse oocytes. The first construct HH-1 contained an about 5,900 bp *HindIII*-*SalI* fragment, including 55 base pairs from the 5' untranslated region and the 5' flanking region of the *LAMC2* gene. The second construct, HH-2, contained 55 base pairs of the 5' untranslated region and 613 base pairs of the 5' flanking region. The sequence from the *LAMC2* promoter region cloned into HH-2 (Fig. 8) contains a GATAA box starting 27 base pairs upstream of the transcription initiation site (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) J. Cell Biol. 119: 679-693), and two AP1 binding sites immediately upstream. Additionally, there are two Sp1 binding sites and an inverted CTF sequence (CCAAT box) further upstream. Three founder lines were studied in detail for the expression pattern of HH-1, and two lines for that of construct HH-2. Both constructs yielded similar expression patterns for the β -galactosidase reporter gene.

In mouse embryos very little expression was observed with both constructs. In 15.5-day-old whole embryos only some hair follicles, testicles and some regions of the skin showed positive reaction (Fig. 9A). Microscopic analysis revealed positive staining in scattered basal keratinocytes of skin and some epithelial cells of hair follicles and *ductus deferens* (Fig. 9B-D). Importantly, cells of all other epithelia were negative for expression. These results sharply contrast our previous results showing strong expression of the *LAMC2* gene in epithelial layers, including those of skin, respiratory tract and kidney in human embryos as determined by *in situ* hybridization (Kallunki, P., Sainio, K., Eddy, R., Byers, M., Kallunki, T., Sariola, H., Beck, K., Hirvonen, H., Shows, T.B. & Tryggvason, K. (1992) J. Cell Biol. 119: 679-693). This shows that the reporter gene constructs made in this study did not contain the *cis*-acting elements necessary for epithelial expression, although the restricted expression observed was limited to only some epithelial cells.

Expression was also examined in tissues of adult transgenic mice harboring the two constructs. Both constructs yielded highly similar, but restricted expression in the embryos, whereas in the adult tissues the distribution of expression was slightly more extensive. Interestingly, as was the case for the embryos, the limited expression was confined to epithelial cells, i.e. cell types normally expressing only the *LAMC2* gene *in vivo*. For example, in skin discontinuous expression was observed in keratinocytes of the epidermis and epithelial cells of some hair follicles (Fig. 10A). In the stomach intense expression could be seen in some villi of the gastric mucosa, but it was absent in most areas (Fig. 10B,C). In positive areas expression could be seen in surface epithelial cells and in cells of the gastric pits. As in the embryos expression was also observed in epithelial cells of the ductus deferens (Fig. 10D). However, epithelia such as those of the respiratory tracts normally showing strong expression *in vivo* did not show any expression at all in the present study.

Identification of a Cis-Element with Migration-Related Activity in the LAMC2 Gene in Transgenic Mice - The *in vitro* cell migration studies described above, together with previous morphological studies have indicated a role for laminin-5 in cell movement. These data imply that the genes for the subunits chains of this protein should have regulatory elements induced during cell migration. In order to further examine the association of cell migration with laminin-5 expression, we decided to initiate search for

potential migration-related *cis*-acting elements in the two laminin $\gamma 2$ chain gene promoter-*LacZ* reporter gene constructs, HH-1 and HH-2, using transgenic mice as a model system.

As we have previously shown, the *LAMC2* gene is expressed in keratinocytes migrating over a healing skin wound (Pyke, C., Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K. & Tryggvason, K (1994) Am. J. Pathol. 145: 782-791). According to the present invention, small incision wounds were made into the dorsal skin and tails of mice made transgenic with the two constructs mentioned above to examine if the *LacZ* gene is expressed in migrating keratinocytes during wound healing. As can be seen in Fig.11, immunostaining of the dorsal skin wound showed distinct linear staining for the laminin $\gamma 2$ chain in the normal subepithelial basement membrane and also strong immunostaining for this chain beneath keratinocytes migrating over the healing wound could be seen. This confirms previous reports that laminin-5 is present in the normal epithelial basement membrane and also that the migrating keratinocytes express laminin-5. Staining with polyclonal antibodies against EHS tumor laminin-1 containing the $\alpha 1$, $\beta 1$ and $\gamma 1$ chains showed a weak staining of the subepithelial basement membrane as well as staining of basement membranes in blood vessels and muscle. Staining for expression of the two β -galactosidase reporter gene constructs showed intense staining in keratinocytes migrating over the healing wound (Fig. 11C,D), while no color reaction was noted in keratinocytes resting on a normal epithelial basement membrane. The staining was seen in several layers of keratinocytes indicating that the enhancer element(s) directs expression in proliferating and migrating keratinocytes. Identical patterns were obtained in mice made with both constructs, demonstrating that the element necessary for driving the expression is located within the 613 base pair region immediately upstream of the transcription site of the *LAMC2* gene.

Thus, according to the present invention, intact laminin-5 effectively mediates attachment of epithelial cells. As set forth above in Example 4, the present invention utilized human keratinocytes and KLN-205 mouse squamous carcinoma cells, both of which were shown to express laminin-5. The effect of laminin-5 on adhesion was similar to that of laminin-1 isolated from the mouse EHS tumor. Both laminin isoforms have been shown to have similar adhesive properties. This adhesion is presumably mediated through $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$ integrins that both bind to the long arm of the laminin molecule.

However, there is also an indication that the short arm of the $\gamma 2$ chain is also involved in the anchorage of epithelial cells, as an in-frame deletion mutation removing 73 amino acid residues from domains III and IV of the short arm of the $\gamma 2$ chain results in lethal (Herlitz) junctional epidermolysis bullosa. In determining whether this particular chain promoted cell adhesion studies were carried out using full-length recombinant human $\gamma 2$ chain produced in insect cells using the baculovirus system. It has been shown by rotary shadowing that individually produced recombinant $\beta 1$ and $\gamma 1$ chains maintain apparent normal tertiary structure of the short arm (Pikkarainen, T., Schulthess, T., Engel, J. and Tryggvason, K.(1992) *Eur. J. Biochem.* 209, 571-582), and, therefore, the tertiary structure of the short arm of the $\gamma 2$ chain studied here was assumed to be normal and exposed. This recombinant chain did not show any significant effects on cell adhesion as compared with plastic. The recombinant chain was not secreted to the cell culture medium, presumably because it was not incorporated into a heterotrimeric molecule and, therefore, it needed to be purified from the cytosol under denaturing conditions, prior to renaturation. Although it is possible that this polypeptide chain lost its adhesive properties due to the denaturing conditions, it is not considered likely, as intact laminin-5, also denatured and renatured using the same conditions, maintained its adhesion activity. Furthermore, since it has been shown that recombinant laminin α chains (or their domains) produced in an eukaryotic expression system bind cellular receptors (Yurchenco, P.D., U. Sung, M. Ward, Y. Yamada & J.J. O'Rear (1993). *J. Biol. Chem.* 268: 8356-8365; Sung, U., J.J. O'Rear & P.D. Yurchenco (1993). *J. Cell Biol.* 123: 1255-1268; Colognato-Pyke, H., J.J. O'Rear, Y. Yamada, S. Carbonetto Y.-S. Cheng, & P. D. Yurchenco (1995). *J. Biol. Chem.* 270: 9398-9406; Colognato, H., M. MacCarrick, J.J. O'Rear & P.D. Yurchenco (1997). *J. Biol. Chem.* 272: 29330-29336), it was concluded that the recombinant $\gamma 2$ chain analyzed in the present study also should be functional. Thus, according to the present invention, the short arm of the laminin $\gamma 2$ chain does not bind to cellular receptors, and the important binding site lost in the above mentioned lethal skin blistering disease interacts with some protein(s) of the extracellular matrix. This explanation is plausible also because it has been shown that the E8 fragment of laminin-1 containing the distal portion of the triple coiled coil (long arm) and recombinant C-terminal G domain of the $\alpha 1$ and $\alpha 2$ chains contains the cell binding sites (Yurchenco, P.D., U. Sung, M. Ward, Y. Yamada & J.J. O'Rear (1993). *J.*

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 S. Cheng, & P. D. Yurchenco (1995). J. Biol. Chem. 270: 9398-9406; Colognato, H., M.
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 5 Aumailley, M., Nurcombe, V., Edgar, D., Paulsson, M. & Timpl, R. (1987) J. Biol.
 Chem. 262: 11532-11539). The laminin binding sites on the epithelial cells have later
 been assigned to the integrins $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$. The conclusion that the short arm of the
 $\gamma 2$ chain does not interact with cellular receptors was further supported by the present
 results showing that polyclonal antibodies raised against the short arm do not inhibit
 10 adhesion of cells to intact laminin-5.

Previous work has shown that expression of laminin-5 is upregulated in
 migrating keratinocytes of healing wounds (Ryan, M.C., Tizard, R., VanDevanter, D.R.
 & Carter, W.G. (1994) J. Biol. Chem. 269: 22779-22787; Larjava, H., Salo, T.,
 Haapasalmi, K., Kramer, R. H. & Heino J. (1993) Clin. Invest. 92: 1425-1435; Pyke, C.,
 15 Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K. & Tryggvason, K (1994)
 Am. J. Pathol. 145: 782-791), and also in tumor cells of invasive carcinomas (Pyke, C.,
 Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K. & Tryggvason, K (1994)
 Am. J. Pathol. 145: 782-791; Pyke, C., Salo, S., Ralfkiaer, E., Romer, J., Dano, K. &
 Tryggvason, K. (1995) Cancer Res. 55: 4132-4139; Tani, T., Karttunen, T., Kiviluoto,
 20 T., Kivilaakso, E., Burgeson, R.E., Sipponen, P. & Virtanen, I. (1996) Am. J. Pathol.
 149: 781-793). Thus, this laminin isoform could not only be surmised to be crucial for
 anchoring epithelial cells to the underlying basement membrane but, additionally, a
 protein of importance for anchoring epithelial cells or epithelium-derived cancer cells to
 the extracellular environment during cell migration. According to the results of Example
 25 4 of the present invention, antibodies against the short arm of the laminin $\gamma 2$ chain
 inhibited the migration of KLN-205 squamous carcinoma cells by about 55 – 65 % as
 determined in the Boyden chamber migration assay. Interestingly, the antibodies used
 here were directed against 177 amino acid residues of domain III that when deleted by
 mutation cause lethal junctional epidermolysis bullosa. Accordingly, the short arm of the
 30 laminin $\gamma 2$ chain is important for the interaction of this laminin isoform to other
 extracellular matrix proteins, and this interaction is also crucial for the migration
 process. Importantly, polyclonal antibodies raised against the long arm of the $\gamma 2$ chain

did not inhibit migration of KLN-205 cells. Consequently, the results of the present study demonstrate that the short arm of the $\gamma 2$ chain is important for the adhesive function of laminin-5 similarly the short arm of the laminin $\gamma 1$ chain that contains the only known binding site of laminins for nidogen. This nidogen binding site is essential for the formation of a bridge between the laminin network on the one hand and the type IV collagen network and perlecan on the other and, thus, it is necessary for the integrity of the entire basement membrane matrix. The present and previous data discussed above indicate that similarly to the $\gamma 1$ chain, the laminin $\gamma 2$ chain possesses an important binding site that anchors laminin-5 to some components of the extracellular matrix. However, the laminin $\gamma 2$ chain does not bind to nidogen .

The results of the experiments shown in Example 4 above with the *LAMC2* promoter-reporter gene constructs further emphasized the involvement of the $\gamma 2$ chain and laminin-5 in cell migration, as the reporter gene was strongly expressed in migratory cells of healing wounds. The actual *cis*-acting element required for this migration-related expression were not identified but it must be located in the 613 base pair upstream region flanking the gene. This region contains several motifs known to be important for gene expression such as a GATAA box, AP-1 and Sp1 binding sites and a CTF motif. However, none of those have been shown to be associated with cell migration. The results of the transgenic mouse experiments carried out in this study further demonstrated that all the enhancer elements necessary for driving normal expression in epithelial cells are not present in a sequence reaching as far as 5,900 bp upstream of the transcription initiation site. Thus far, no other tissue-specific enhancer elements have been reported for any laminin gene.

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.